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(54) Title: PROINSULIN-LIKE COMPOUNDS

(57) Abstract

Compounds consisting of insulin or an insulin analogue in which the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the A-chain by a connecting peptide containing 1 through 15 amino acid residues in which the C-terminal amino acid residue is different from Lys and Arg, have advantageous effects, e.g., in the treatment of diabetics.

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PROINSULIN-LIKE COMPOUNDS

FIELD OF THIS INVENTION

This invention relates to proinsulin-like compounds, a method for producing such compounds, and pharmaceutical compositions containing such compounds.

BACKGROUND OF THIS INVENTION

The polypeptide hormone insulin is a 51 amino acid protein consisting of an A chain with 21 amino acids and a B-chain with 30 amino acids, the A- and B- chains being interconnected by disulphide bridges. Insulin is essential in maintaining normal metabolic regulation.

The polypeptide hormone insulin-like growth factor I (IGF-I) (also called somatomedin C) is a 70 amino acid protein involved in mediating many effects of growth hormone. IGF-I has been demonstrated to stimulate growth in hypophysectomized rats as well as promoting cell growth and differentiation of various cell types.

Insulin and IGF-I are highly homologous in amino acids sequence; in particular, all cysteines are conserved between insulin and IGF-I. One of the major differences between insulin and IGF-I is that normally all (more than 99%) IGF-I present in human blood is found in association with special serum carrier proteins which do not readily cross the capillary barrier. The role of the IGF-I binding proteins is not clear, but they clearly play a role in modulating the activity of IGF-I, e.g., the major part of IGF-I in the serum is inactive. In contrast to IGF-I, insulin does not associate with specific carrier proteins, or with the IGF-I carrier proteins.

The polypeptide hormone insulin binds with high affinity to the insulin

receptor and the polypeptide hormone IGF-I binds to the IGF-I receptor with high affinity. In contrast, insulin binds to the IGF-I receptor with an affinity being 100 - 1000 fold lower than to the insulin receptor, likewise IGF-I binds to the insulin receptor with an affinity being 100 - 1000 fold lower than to the IGF-I receptor (Kjeldsen et al.). The insulin receptor and the IGF-I receptor show extensive similarity in amino acids sequence, domain structure and signalling mechanism.

The two chain structure of insulin allows insulin to undertake multiple conformations, and several findings have indicated that insulin have the propensity to considerable conformational change and that restrictions in the potential for such change considerably decrease the affinity of insulin receptors for ligand. Conformational restrictions in the insulin molecule have been shown in several ways to significantly decrease the affinity of receptor for ligand. Proinsulin has a 100 fold lower affinity for the insulin receptor than native insulin (Nakagawa & Tager). Blocking of the amino acid residue A1 in insulin also results in poor receptor binding, consistent with the dogma that a free N-terminal of the A-chain and free C-terminal of the B-chain of insulin are important for binding to the insulin receptor.

IGF-I has been shown to lower the blood glucose level in various animals including man (Turkalj et al.), and IGF-I is capable of imitating the metabolic effects of insulin. The short term hypoglycaemic responses to bolus injection of insulin and IGF-I are identical in healthy adults, when correcting the doses administered for the lower potency of IGF-I (6 % of insulin) (Guler et al.).

Insulin resistance is defined as a subnormal biological response to a given concentration of insulin. Some of the most severe cases of insulin resistance are observed in patients with mutations in the insulin receptor. In addition, insulin resistance is likely to be the first mechanism which leads to type 2 diabetes. Insulin resistance results in deficient insulin action, and treatment of insulin resistance with insulin is not effective. Today, the mostly used compounds, specific of insulin resistance, are biguanides which sensitise peripheral tissue to the action of insulin. However, the efficacy of these drugs is moderate and

limited to type 2 diabetes (Vialettes). Consequently, new drugs for treating the insulin resistance accompanying types 1 and 2 diabetes are needed.

One object of this invention is to furnish compounds which effectively can be used to treat diabetics, preferably patients with insulin resistance.

A further object of this invention is to furnish compounds for treating other metabolic disorders that could favour from exogenic administration of a compound that binds to the insulin receptor as well as the IGF-I receptor, e.g., leprechaunism, and lipodysthrophy.

A still further object of this invention is to provide pharmaceutical compositions containing such compounds.

BRIEF DESCRIPTION OF THIS INVENTION

It has now, surprisingly, been found that compounds consisting of insulin or an insulin analogue in which the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the A-chain by a connecting peptide containing 1 through 15 amino acid residues in which the C-terminal amino acid residue is different from Lys and Arg, have advantageous effects. Especially, these compounds can effectively be used to treat diabetics, preferably patients with insulin resistance. In contrast to what was previously believed about the structure/function relationship of the insulin receptor binding of insulin and insulin analogs, it is especially surprising that the compounds of this invention bind with high affinity to the insulin receptor. Additionally, it is surprising that these compounds also bind to the IGF-I receptor.

DETAILED DESCRIPTION OF THIS INVENTION

The compounds of this invention are insulin wherein the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the

A-chain via a connecting peptide or are insulin analogues wherein the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the A-chain via a connecting peptide.

The term insulin, when used alone, covers natural occuring insulins such as human insulin, porcine insulin and bovine insulin, human insulin being preferred.

Examples of insulin derivatives are insulins wherein one or more of the amino acid residues in positions 9, 16, 28 and 29 of the B-chain of insulin have been substituted with another amino acid residue. Preferably, these amino acids are those amino acids which can be coded for by the nucleotide sequences. Examples of such insulin derivatives are insulins containing Asp^{B9}, Glu^{B16}, Glu^{B28}, Lys^{B28}, Pro^{B28} and/or Pro^{B29}. Examples of other insulin analogues are insulins wherein the B29 lysine residue is bound to a lipophilic group via its epsilion amino group. This lipophilic group may be an acyl group containing 6 through 24 carbon atoms. An example of other insulin analogues is insulins wherein the A21 asparagine residue is exchanged with another amino acid residue. Preferably, these amino acid residues corresponds to those amino acids which can be coded for by the nucleotide sequences.

The connecting peptide is a peptide moiety connecting the C-terminal amino acid residue of the B-chain with the N-terminal amino acid residue in the A-chain of insulin or of an insulin analogue. According to this invention, the connecting peptide present in the compounds of this invention contains 1 through 15 amino acid residues. Preferably, these amino acid residues corresponds to those amino acids which can be coded for by the nucleotide sequences. In this connecting peptide, the C terminal amino acid residue is different from lysine (Lys) and arginine (Arg). In a preferred embodimenet of this invention, the connecting peptide contains 9 - 15 amino acid residues, and more preferred it contains 12 amino acid residues.

Examples of two specific connecting peptides are the peptide residue GYGSSSRRAPQT (designated by the one letter codes for the amino acids) and GYGSSSAAAPQT. In the sequence listing below, these two peptide residues are

SEQ ID No. 1 and 2, respectively. In these sequences, G (Gly) is to be connected to the C terminal end of the B chain in insulin or in the insulin analogue. Indicated by the three letter codes for amino acids, these two peptide residues have the formula -Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-Ala-Pro-Gln-Thr- and -Gly-Tyr-Gly-Ser-Ser-Ser-Ala-Ala-Ala-Pro-Gln-Thr-, respectively. Examples of further preferred connecting peptides are the peptide residues GYGSSSRRAPQT or GYGSSSAAAPQT (designated by the one letter codes for the amino acids) from which some of the amino acid residues have been deleted or exchanged with other amino acid residues, the number of deleted or exchanged amino acid residues preferably being not more than 6 residues, more preferred being not more than 4 residues, and most preferred not more than 2 residues have been deleted or exchanged.

The compounds of this invention can be prepared by a manner known per se. For example, the compounds of this invention can be prepared by the recombinant DNA expression systems of bacteria, yeast or tissue cell culture host which comprises:

a) insertion of the appropriate synthetic gene into an expression vector to form an expression cassette;

b) introduction of the expression cassette into the bacteria, yeast or tissue culture host;

c) growth of the transformed expression host; and

d) purification of the desired polypeptide analog from said host.

A more specific way of doing this is to prepare a synthetic gene encoding a compound of this invention or an extended precursor thereof, for example by overlap extension PCR techniques (Polymerase Chain Reaction) using primers covering the full length sequence. The resulting PCR fragment is digested with suitable restriction enzymes and ligated into a yeast expression vector furnished with a synthetic leader sequence. The vector is introduced into a yeast strain, for example a Saccharomyces cerevisiae strain. The yeast strain is grown in a suitable medium. Thereafter, the compound or the precursor is isolated using

suitable purification methods and, if necessary, extended precursors are converted to the desired compounds.

The compounds of this invention can also be prepared by culturing a yeast strain containing a replicable expression vector comprising a DNA-sequence encoding a compound according to this invention in a suitable nutrient medium, and then recovering the compound from the culture medium.

The compounds of this invention can be used for the composition of novel insulin compositions. These novel insulin compositions can be used instead of the insulin compositions heretofore known to the art. Such novel insulin compositions contain a compound according to this invention or a pharmaceutically acceptable salt thereof in aqueous solution or suspension, preferably at neutral pH. The aqueous medium is made isotonic, for example, with sodium chloride, sodium acetate or glycerol. Furthermore, the aqueous medium may contain zinc ions, buffers such as acetate and citrate and preservatives such as m-cresol, methylparaben or phenol. The pH value of the composition is adjusted to the desired value and the insulin composition is made sterile by sterile filtration. Consequently, this invention also relates to a pharmaceutical composition containing a compound of this invention and, optionally, one or more agents suitable for stabilization, preservation or isotoni, for example, zinc ions, phenol, cresol, a parabene, sodium chloride, glycerol or mannitol.

The compounds of this invention may also be mixed with other insulins or insulin analogues having a protracted insulin activity to prepare insulin compositions consisting of a mixture of rapid acting and protracted insulin.

The insulin compositions of this invention can be used similarly to the use of the known insulin compositions for the treatment of mammals, preferably man, suffering from diabetes. The daily dose to be administered in therapy can be determined by a physician and will, inter alia, depend on the particular com-

pound employed and on the condition of the patient. Usually, the compositions of this invention are administered subcutaneously.

The abbreviations used for the amino acids are those stated in J.Biol.Chem. 243 (1968), 3558.

Any novel feature or combination of features described herein is considered essential to this invention.

This invention is further illustrated by the following examples which, however, are not to be construed as limiting.

EXAMPLE 1

Construction of the gene

A synthetic gene encoding for human insulin in which the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the A-chain by the peptide residue GYGSSSRRAPQT wherein Gly (G) is connected to B30 (hereinafter designated ICP), was constructed by overlap extension PCR techniques using two primers covering the full length sequence. The resulting PCR fragment was digested with NcoI and XbaI and ligated into the NcoI/XbaI site of a cPOT yeast expression vector furnished with a synthetic leader sequence. The 170 bp HindIII/XbaI fragment of ICP was subcloned into the corresponding site of a cPOT vector furnished with the α-leader sequence.

Transformation of E.Coli with the ligation mixture yielded bacteria carrying the plasmid. The compound which it encodes is shown in the sequence listing below as SEQ ID No. 3.

Expression and purification

Saccharomyces cerevisiae strain MT663 was transformed with the expression plasmid and transformants were selected on YPD plates. Cells were grown to saturation in 1 liter of YPD with 5 mM $CaCl_2$. The secreted ICP compound was purified from the conditioned media by three steps. Initially, the media was adjusted to a pH value of 3 with HCl and batch treated with Lewatit® 120 to adsorb peptides which were subsequently eluted with 0.5 M ammonium. The ICP compound was purified by reverse phase HPLC (high pressure liquid chromatography) on a LiChrosorb® column. The sample was desalted using a PD10 column, and finally, the ammonium was removed by drying the eluate. The purified polypeptide was characterized by mass spectroscopy, N-terminal sequencing, immuno blotting, and silver staining of tricine SDS-PAGE gels. The quantity of polypeptide was determined by HPLC.

EXAMPLE 2

Receptor preparation and binding assays

The ICP compound was characterized by binding to truncated insulin and IGF-I receptors.

Synthetic genes encoding truncated insulin receptors and truncated IGF-I receptors were constructed from a full legth receptor cDNA using synthetic oligonucleotide linkers and overlap extension by polymerase chain reaction (Perkin Elmer, Cetus).

cDNA encoding truncated receptors were inserted into the mammalian expression vector pZem. Inserted cDNA fragments and junctional regions were sequenced using enzymatic chain termination. Expression vectors encoding the truncated receptors were stably transfected into baby hamster kidney cells (BHK) and individual clones expressing the recombinant receptors were selected

as described (Andersen).

Soluble truncated insulin and IGF-I receptors secreted from the transfected BHK cells were partially purified by the previously described procedure (Kjeldsen et al.). Culture medium (12.5 ml) was diluted with one volume 20 mM Tris-HCl (pH 8.0) and applied to a 1 ml Q Sepharose® Fast Flow column (Pharmacia). Bound material was eluted with a gradient from 0 - 500 mM NaCl in 20 mM Tris-HCl (pH 8.0) over 15 minutes, running at 1 ml/min. Fractions containing binding activity were concentrated on Centricon-100 microconcentrators (Amicon) and applied on a Superose 6 column (Pharmacia), running in 25 mM Hepes (pH 8.0), 100 mM NaCl at 0.5 ml/min. Eluted receptors were stored at -20°C.

Competition binding assays were performed by incubating the receptors in a total volume of 200 µl with ^{125}I-IGF-I (10 pM) (Amersham) and increasing concentrations of unlabeled ligand in 100 mM Hepes (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5% BSA (bovine serum albumin), 0.025% Triton® X-100 for 48 hours at 4°C. Subsequently, bound ligand was precipitated with 0.2 % gammaglobulin and 500 µl of 25 % PEG 8000 (polyethyleneglycol), and the radioactivity in the pellet was counted. The concentration of the receptors was adjusted to yield 15 - 20 % binding when no competing ligand was added in the competition assay.

The competition binding data were analysed according to a four parameter logistic equation to determine IC_{50} values using GraFit® software. IC_{50} is defined as the concentration of ligand needed to bring about 50 % inhibition of tracer (^{125}I labeled) binding to the receptor.

Relative binding to soluble receptors

IC_{50} values of insulin, IGF-I and ICP relative to cognate ligand.

Ligand	Insulin	IGF-I	ICP
Insulin receptor	100 %	7 %	100 %
IGF-I receptor	0.1 %	100 %	21 %

EXAMPLE 3

Receptor binding experiment.

The ICP polypeptide was characterized by binding to insulin and IGF-I receptors.

Synthetic genes encoding truncated insulin receptors and truncated IGF-I receptors were constructed from full length receptor cDNA using synthetic oligonucleotide linkers and overlap extension by polymerase chain reaction (Perkin Elmer, Cetus).

cDNA encoding truncated and holo-receptors was inserted into the mammalian expression vector pZem. Inserted cDNA fragments and junctional regions were sequenced using enzymatic chain termination. Expression vectors encoding the receptors were stably transfected into baby hamster kidney cells (BHK) and individual clones expressing the recombinant receptors were selected as described (Andersen et al.).

Soluble truncated insulin and IGF-I receptors secreted from the transfected BHK cells were partially purified by the previously described procedure (Kjeldsen et al.). Culture medium (12.5 ml) was diluted with one volume 20 mM Tris-HCl (pH 8.0) and applied to a 1 ml Q Sepharose Fast Flow column (Pharmacia). Bound material was eluted with a gradient from 0-500 mM NaCl in 20 mM Tris-HCl (pH 8.0) over 15 min, running at 1 ml/min. Fractions containing binding activity were concentrated on Centricon-100 microconcentrators (Amicon) and applied on a Superose 6 column (Pharmacia), running in 25 mM Hepes (pH 8.0), 100 mM NaCl at 0.5 ml/min. Eluted receptors were stored at -80°C.

Competition binding assays using soluble truncated receptors were performed by incubating the receptors in a total volume of 0.2 ml with ^{125}I-IGF-I (10 pM) (Amersham) and increasing concentrations of unlabeled ligand in 100 mM Hepes (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 % BSA, 0.025 % Triton X-

100 for 48 hours at 4°C. Subsequently, bound ligand was precipitated with 0.2 % gammaglobulin and 0.5 ml 25 % PEG 8000, and the radioactivity in the pellet was counted. The concentration of the receptors were adjusted to yield 15 - 20 % binding when no competing ligand was added in the competition assay.

Competition binding assays on membrane-bound holo receptors were performed on BHK cells overexpressing full length insulin (-exon11) or IGF-I receptors. Equal number of transfected BHK cells (2000 - 5000) was seeded in each well of a 24 well plate and grown for 24 hours in Dulbecco's modified Eagle's medium (Lifetech) containing 10 % fetal calf serum (Lifetech) before performing binding assay. Cells were washed once with binding buffer B (Dulbecco Modified Eagle Medium, 0.5 % BSA, 20 mM Hepes (pH value: 7.8)) before adding a total volume of 0.4 ml with ^{125}I-IGF-I (6.5 pM) or ^{125}I-insulin (6.5 pM) and increasing concentrations of unlabeled ligand in binding buffer B. After 3 hours at 16°C, unbound ligand was removed by aspirating the buffer and washing once with 1.2 ml of cold binding buffer B, cells were solubilized in 0.5 ml 1 % SDS, 100 mM NaCl, 25 mM Hepes (pH 7.8) and counted. The number of cells was adjusted to yield 16 - 28 % binding when no competing ligand was added in the assay.

The competition binding data were analysed according to a four parameter logistic equation to determine IC_{50} values using GraFit software.

Relative binding to soluble receptors

IC_{50} values of insulin, IGF-I and ICP relative to cognate ligand.

	Insulin	IGF-I	ICP
Insulin rec.	100 %	9 %	94 %
IGF-I rec.	0.2 %	100 %	19 %

Relative binding to membrane bound holo-receptors

IC_{50} values of insulin, IGF-I and ICP relative to cognate ligand.

Ligand	Insulin	IGF-I	ICP
Insulin rec.	100 %	1 %	113 %
IGF-I rec.	0.1 %	100 %	28 %

EXAMPLE 4

In vivo effects of ICP.

It was the aim to measure the hypoglycaemic efficacy of ICP in normal rats. In order to evaluate the potency of ICP, the effect of two different dose levels were tested and compared with the effect of two different dose levels of human insulin (hereinafter designated HI).

Method :

Thirty male sprague dawley rats of a body weight (hereinafter designated BW) in the range 200 - 220 g were fasted for 18 hours prior to the experiment. The animals were randomised into 5 different groups (n = 6 per group). These groups were as follows:

A: ICP, 6.2 nmoles/kg; B: ICP, 18.6 nmoles/kg; C: HI, 2.1 nmoles/kg; D: HI, 6.2 nmoles/kg; and E: Control.

The test substance was administered as a single subcutaneous injection in a volume of 0.5 ml/250 g BW. ICP and HI were dissolved in 0.01 M HCl and diluted with saline till the final concentration was reached (pH value: 6.4 - 7.1). Control animals were injected with vehicle.

Blood glucose (hereinafter designated BG) was measured by a glucose oxidase method in an autoanalyzer (EBIO 6666) in samples of 10 µl of blood obtained from the tail vasculature at 0, 30, 60, 90 and 120 minutes.

Results :

In group A, BG decreased from basal level 5.1 ± 0.3 mmol/l (0 min) to 2.8 ± 0.4 mmol/l at peak effect (60 minutes).

In group B, BG decreased from basal level 5.1 ± 0.3 mmol/l (0 min) to 2.3 ± 0.2 mmol/l at peak effect (60 minutes).

In group C, BG decreased from basal level 5.0 ± 0.3 mmol/l (0 min) to 3.5 ± 0.3 mmol/l at peak effect (60 minutes).

In group D, BG decreased from basal level 4.8 ± 0.3 mmol/l (0 min) to 2.2 ± 0.4 mmol/l at peak effect (90 minutes).

In group E (control), BG decreased from basal level 5.1 ± 0.2 mmol/l to 4.5 ± 0.3 mmol/l at 60 minutes.

All treatment groups (A - D) differed significantly from the corresponding control value at 60 minutes ($p < 0.001$).

Calculated total $AUC_{glucose}$ for all groups

Group	$AUC_{glucose}$ mmol/l/120 minutes	Decrease relative to control %
A	416 ± 27 *	26
B	316 ± 12 *	44
C	491 ± 27 *	12
D	330 ± 27 *	41
E	560 ± 22	0

All values are means ± sd (hereinafter standard deviation). AUC is an abbreviation for the "Area Under the Curve" which indicates the insulin lowering effect.

* significantly different from control (E), $p < 0.001$

It is concluded that ICP exhibits hypoglycaemic effect in normal rats after a single subcutaneous injection. The efficacy of ICP is similar to the efficacy of HI, and the potency of ICP is between 33 % and 100 % of the potency of HI.

References:

Turkalj et al.: J.Clin.Endocrin.Metab. 75 (1992), 1186 - 1191.

Guler et al.: New England J.Med. 317 (1987), 137 - 140.

Kjeldsen et al.: Proc.Natl.Acad.Sci.USA 88 (1991), 4404 - 4408.

Nakagawa & Tager: Biochemistry 32 (1993), 7237 - 7243.

Vialettes: Horm.Res. 38 (1992), 51 - 56.

Andersen et al.: Biochemistry 29 (1990), 7363 - 7366.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(I) TELEX: 37173

(ii) TITLE OF INVENTION: PROINSULIN-LIKE COMPOUNDS

(iii) NUMBER OF SEQUENCES: 3

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: DK 1400/93
(B) FILING DATE: 17-DEC-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: DK 1399/93
(B) FILING DATE: 17-DEC-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: DK 0029/94
(B) FILING DATE: 07-JAN-1994

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Tyr Gly Ser Ser Ser Arg Arg Ala Pro Gln Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Tyr Gly Ser Ser Ser Ala Ala Ala Pro Gln Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Gly Tyr
20 25 30

Gly Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Glu Gln Cys
35 40 45

Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
50 55 60

CLAIMS

1. Compounds consisting of insulin or an insulin analogue in which the C-terminal amino acid residue of the B-chain is connected with the N-terminal amino acid residue in the A-chain via a peptide chain (a connecting peptide) containing 1 through 15 amino acid residues, with the proviso that the C-terminal amino acid residue in this connecting peptide is different from Lys and Arg.

2. Compounds, according to Claim 1, wherein the insulin analog is insulin wherein one or more of the amino acid residues in positions 9, 16, 28 and 29 of the B-chain have been exchanged with another amino acid residue, preferably compounds wherein the B chain contains Asp^{B9}, Glu^{B16}, Glu^{B28}, Lys^{B28}, Pro^{B28} and/or Pro^{B29}.

3. Compounds, according to any one of the preceding claims, wherein the insulin analog is insulin wherein one of the amino acid residues in positions 24, 25, 26, 27 and 28 of the B-chain has been deleted.

4. Compounds, according to any one of the preceding claims, wherein the insulin analog is insulin wherein the B29 lysine residue is bound to a lipophilic group via its epsilion amino group, and preferably this lipophilic group is an acyl group having 6 through 24 carbon atoms.

5. Compounds, according to any one of the preceding claims, wherein the A21 asparagine residue is exchanged with another amino acid residue.

6. Compounds, according to Claim 1, consisting of natural occurring insulin, preferably human insulin, and the connecting peptide.

7. Compounds, according to any one of preceding claims, wherein the connecting peptide contains 9 through 15 amino acid residues, preferably 12 amino acid residues.

8. Compounds, according to the preceding claim, wherein the connecting peptide is GYGSSSRRAPQT (designated by the one letter codes for the amino acids) or GYGSSSAAAPQT.

9. Compounds, according to any one of the preceding claims, wherein the connecting peptide is the peptide residue GYGSSSRRAPQT or GYGSSSAAAPQT in which at the most 6 of the amino acid residues have been cancelled or exchanged with other amino acid residues, preferably at the most 4 of the amino acid residues have been cancelled or exchanged with other amino acid residues, and most preferred at the most 2 of the amino acid residues have been cancelled or exchanged with other amino acid residues.

10. Compounds, according to any one of the Claims 2, 5, 7 and 9, wherein the amino acid residue is an amino acid residue which can be coded for by a triplet of nucleotides.

11. Synthetic genes encoding for the compounds according to any one of the preceding claims.

12. A process for the preparation of a compound according to any one of Claims 1 through 10 by the recombinant DNA expression systems of bacteria, yeast or tissue cell culture host which comprises:

a) insertion of the appropriate synthetic gene into an expression vector to form an expression cassette;

b) introduction of the expression cassette into the bacteria, yeast or tissue culture host;

c) cultivation of the transformed expression host; and

d) purification of the desired polypeptide analog from said host.

13. A method for the production of a compound according to any one of Claims 1 through 10, wherein a yeast strain containing a replicable expression vehicle comprising a DNA-sequence encoding for a gene according to Claim 11 is cultured in a suitable nutrient medium, and the compound is recovered from the culture medium.

14. A method according to the preceding claim, in which the yeast is Saccharomyces cerevisiae.

15. A method for treating or preventing hyperglycemia in mammals, preferably man, which comprises administering an effective amount of a compound according to any one of the Claims 1 through 10.

16. A method for treating hyperlipidaemia, growth disorders, or wounds in mammals, preferably man, by administering an effective amount of a compound according to any one of Claims 1 through 10.

17. A pharmaceutical composition characterised in that it contains a compound according to any one of Claims 1 through 10 and a suitable carrier such as one or more agents suitable for stabilization, preservation or isotoni, for example, zinc ions, phenol, cresol, a parabene, sodium chloride, glycerol or mannitol.

18. A pharmaceutical composition according to the preceding claim, also containing insulin.

19. Injectable solutions with an insulin activity, characterised in that they contain an effective amount of a compound according to any one of Claims 1 through 10 or a pharmaceutically acceptable salt thereof in aqueous solution,

preferably having a pH value around neutral pH.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00471

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, C12N 15/17, A61K 38/28
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, WPI, WPIL, SCISEARCH, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Service, file 155, Medline, Dialog accesion No. 06612186, Medline accession No. 88257186, Powell SK et al: "Efficient targeting to storage granules of human proinsulins with altered propeptide domain", J Cell Biol (UNITED STATES) Jun 1988, 106 (6) p 1843-51 --	1-14,17-19
X	EP, A2, 0347781 (HOECHST AKTIENGESELLSCHAFT), 27 December 1989 (27.12.89), page 2, line 54 - page 3, line 2, the claims --	1-14,17-19
A	WO, A1, 9323067 (THOMAS JEFFERSON UNIVERSITY), 25 November 1993 (25.11.93), page 5, line 30 - line 31 --	1-14,17-19

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 March 1995	29 -03- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00471

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9102807 (AMGEN INC.), 7 March 1991 (07.03.91), page 2, line 19 - line 29 --	1-14,17-19
A	WO, A1, 9001038 (NORDISK GENTOFTE A/S), 8 February 1990 (08.02.90), the abstract --	1-14,17-19
A	WO, A1, 8806599 (NOVO INDUSTRI A/S), 7 Sept 1988 (07.09.88) --	1-14,17-19
A	GB, A, 1285023 (NOVO TERAPEUTISK LABORATORIUM A/S), 9 August 1972 (09.08.72), the claims --	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00471

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15-16
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1 (iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

25/02/95

International application No.
PCT/DK 94/00471

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0347781	27/12/89	SE-T3- 0347781	
		AU-B- 611303	06/06/91
		AU-A- 3671889	04/01/90
		DE-A- 3919852	28/12/89
		DE-D- 58906966	00/00/00
		ES-T- 2061797	16/12/94
		JP-A- 2085298	26/03/90
		PT-B- 90939	30/11/94
WO-A1- 9323067	25/11/93	NONE	
WO-A1- 9102807	07/03/91	AU-B- 644105	02/12/93
		AU-A- 6425490	03/04/91
		CA-A- 2023969	26/02/91
		EP-A,A- 0441955	21/08/91
		JP-T- 4501514	19/03/92
		US-A- 5158875	27/10/92
WO-A1- 9001038	08/02/90	AU-B- 616205	24/10/91
		AU-A- 2263688	19/02/90
		EP-A,B- 0425482	08/05/91
		ES-A- 2010299	01/11/89
		US-A- 5149777	22/09/92
WO-A1- 8806599	07/09/88	AU-B- 612141	04/07/91
		AU-A- 1397688	26/09/88
		DE-A- 3880346	27/05/93
		EP-A,A,A 0280534	31/08/88
		SE-T3- 0280534	
		ES-T- 2053723	01/08/94
		JP-T- 1502434	24/08/89
		US-A- 5008241	16/04/91
		ZA-A- 8801280	23/08/88
		AU-B- 616381	31/10/91
		AU-A- 7990787	21/04/88
		CA-A- 1316819	27/04/93
		DE-A- 3778287	21/05/92
		EP-A,A,A 0265213	27/04/88
		SE-T3- 0265213	
		IE-B- 60264	29/06/94
		US-A- 5070186	03/12/91
GB-A- 1285023	09/08/72	AT-B- 354634	25/01/79
		BE-A- 737257	09/02/70
		CH-A- 526958	31/08/72
		DE-A,C- 1940130	12/02/70
		FR-A- 2015369	24/04/70
		NL-A- 6912142	11/02/70